Retinol Status and Expression of Retinol-Related Proteins in Methionine-Choline Deficient Rats

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Summary Retinol and its derivative, retinoic acid, have pleiotropic functions including vision, immunity, hematopoiesis, reproduction, cell differentiation/growth, and development. Non-alcoholic fatty liver disease (NAFLD) is one of the most common diseases in developed countries and encompasses a broad spectrum of forms, ranging from steatosis to steatohepatitis, which develops further to cirrhosis. Retinol status has an important role in liver homeostasis. The purpose of this study was to evaluate the retinol status and expression of retinol-related proteins, including enzymes and binding proteins, in methionine-choline deficient (MCD) rats as a model of NAFLD. We examined retinol levels in the plasma and liver and gene expression for \(\beta\)-carotene 15,15′-monooxygenase (BCMO), lecithin:retinol acyltransferase (LRAT), aldehyde dehydrogenase 1A1 (ALDH1A1), ALDH1A2, and cellular retinol binding protein (CRBP)-I in MCD rats. The plasma retinol levels in MCD rats were lower than those in the controls, whereas hepatic retinol levels in MCD rats were higher. BCMO expression in the intestine and liver in MCD rats was lower, whereas that in the testes and the kidneys was higher than in control rats. Expression of LRAT, CRBP-I, ALDH1A1, and ALDH1A2 in the liver of MCD rats was also higher. Altered expression of retinol-related proteins may affect retinol status in NAFLD.

Key Words retinol, non-alcoholic fatty liver disease, \(\beta\)-carotene 15,15′-monooxygenase

Vitamin A (retinol) plays an important role in vision and has pleiotropic functions in immune response, hematopoiesis, fertility, cell differentiation, growth, and morphogenesis (1). Both retinol deficiency and excessive doses of retinol cause malformations in the embryos of many vertebrate species, demonstrating that retinol plays an important role in embryogenesis. Retinoic acids, derivatives of retinol, act as the ligands of retinoic acid receptors (RAR) and retinoid X receptors (RXR), which are members of the nuclear receptor superfamily and are ligand-dependent transcriptional factors (2). RARs and RXRs have subtypes (\(\alpha\), \(\beta\), and \(\gamma\)), and 9-cis retinoic acid (an isomer of retinoic acid) binds RARs and RXRs, whereas all-trans retinoic acid binds only RARs. Both types of retinoic acid receptors transcriptionally regulate the expression of target genes and exert multiple physiological functions.

Dietary retinol has 2 major forms: retinyl esters derived from animal sources and provitamin A carotenoids, mainly in the form of \(\beta\)-carotene, derived from plants (3, 4). Dietary retinyl esters are emulsified with fatty acids and bile salts and then micellized before hydrolysis in the lumen of the small intestine for retinol uptake. In the enterocytes, retinol is rapidly re-esterified with long-chain fatty acids. \(\beta\)-Carotene is absorbed in intestinal mucosal cells and then cleaved for retinal formation. \(\beta\)-Carotene 15,15′-monooxygenase (BCMO), the key enzyme of this process, cleaves \(\beta\)-carotene into 2 molecules of retinal, which are then esterified to retinyl esters in the intestine (4). Retinyl esters that are incorporated into chylomicrons are secreted from enterocytes and transferred to the liver through the lymphatic circulation. Chylomicrons containing retinyl esters are taken up by the liver and transferred to hepatic stellate cells (HSCs) for storage. Retinol in the liver is esterified by the enzyme lecithin:retinol acyltransferase (LRAT) to form retinyl esters. The transfer of retinol in the liver is mediated by cellular retinol binding protein (CRBP)-I, which delivers retinol to retinol binding protein (RBP) for secretion into the circulation. Retinoic acid levels are tightly regulated at the biosynthesis and oxidation stages (3). Retinol is reversibly oxidized to retinal by retinol dehydrogenases, and retinal is irreversibly oxidized to retinoic acid by members of the retinal dehydrogenase (ALDH) family. As mentioned above, the biological actions of retinoic acid isomers (all-trans and 9-cis forms) are conducted through their role as RAR and RXR ligands. Retinoic acids are oxidized by the cytochrome P450 26 family (CYP26A1, CYP26B1, and CYP26C1), following which their metabolites are conjugated with glucuronic acid.

Non-alcoholic fatty liver disease (NAFLD) is one of the most common diseases in developed countries, and
its presentation ranges from steatosis to steatohepatitis or non-alcoholic steatohepatitis (NASH), which further develops to cirrhosis. The definition of NASH includes inflammation of the liver, ballooning hepatocytes, necroapoptosis, and fibrosis (5). The major risk factors of NAFLD are obesity with insulin resistance, type 2 diabetes, dyslipidemia, and metabolic syndrome. There are a number of clinical trials relating to the treatment of NAFLD, including dietary changes with or without exercise and pharmacological treatment (5, 6). As an initial intervention, dietary change in combination with exercise is effective for NAFLD and leads to weight loss-related improvements in aminotransferase levels and liver histology. From the standpoint of cell signaling, exercise influences peroxisome proliferator-activated receptor (PPAR) signaling, mitochondrion metabolism, skeletal muscle substrate utilization, and insulin signaling (5). Several pharmacological treatments for NAFLD have been explored in human clinical studies. Clinical trials for NASH include the use of anti-diabetic agents (pioglitazone, rosiglitazone, and metformin), cytoprotective agents (ursodeoxycholic acid and betaine), and other agents (simvastatin and losartan) (5, 6). Of all of these, vitamin E antioxidant therapy and/or statins may be effective options for NAFLD treatment.

Retinol and its derivates retinoic acid have critical roles in liver regeneration and pathogenesis including inflammation, steatosis, fibrosis, cirrhosis, and cancer (7). HSCs store retinol as retinyl palmitate in lipid droplets and contain CRBPs, cellular retinoic acid binding proteins, and retinol-metabolizing enzymes. HSC dysfunction is related to the pathogenesis and development of liver disease. Transgenic mice exhibiting the dominant negative RARα in a hepatocyte-specific manner developed steatohepatitis and finally hepatocellular carcinoma (8). Moreover, accelerated reactive oxygen species were observed, leading to death and proliferation of hepatocytes (9). Therefore, retinol status and retinol-related protein expression patterns in the liver and other tissues might be involved in NAFLD pathogenesis. In the current study, we examined the retinol status and expression of hepatic retinol-related proteins, including binding proteins and metabolizing enzymes, in NAFLD model rats as well as the relationship between retinol status and the expression of these proteins in NAFLD.

**MATERIALS AND METHODS**

**Animal experiments.** Wistar rats (male, aged 4 wk) were purchased from Japan SLC, Inc. (Shizuoka, Japan) for this study. Two groups of 6 rats were given free access to the following diets: the standard diet (control group) and the methionine-choline deficient diet (MCD group). Each diet was purchased from Funahashi Farm (Chiba, Japan), and each diet contained retinol (1,000 IU/100 g) and β-carotene (0.02 mg/kg). After 6 wk, the rats were sacrificed by exsanguination under isoflurane anesthesia after an overnight fast. Blood was collected in heparinized tubes, and plasma was separated for storage at −80°C. The liver, intestine, testes, and kidneys were removed, immediately frozen in liquid nitrogen, and stored at −80°C. A portion of the liver tissue was processed to make paraffin sections. Care and handling of the experimental animals were done according to Osaka Medical College guidelines for the ethical treatment of laboratory animals.

**Biochemical analysis.** The plasma levels of total lipid, triglycerides, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were measured using enzyme colorimetric assays. Thiobarbituric acid-reactive substances (TBARS) in the liver tissues were measured, as previously described (10). The retinol levels in the plasma and liver homogenates were assayed using high-performance liquid chromatography, as described previously (11). For analysis, liver tissue was homogenized and saponified with one-twentieth volume of 60% potassium hydroxide in distilled water, after which the saponified liver samples were extracted with hexane. The protein content was then measured according to the method of Bradford (12).

**Hematoxylin and eosin staining.** Liver tissue sections were stained with hematoxylin and eosin (H&E) for observation using a light microscope.

**Immunoblotting.** Anti-rat LRAT antibody (Immuno-Biological Laboratories, Gunma, Japan), anti-CRBP-I antibody (Santa Cruz Biotechnology Inc., Santa Cruz, USA), and anti-CRBP-II antibody (Santa Cruz Biotechnology Inc., Santa Cruz, USA) were used. All primary antibodies were used at 1:100 dilution. As a negative control, no antibody was used. Membranes were incubated in 5% milk in Tris-buffered saline overnight. Membranes were then washed in Tris-buffered saline three times and incubated in horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h. Membranes were then washed three times in Tris-buffered saline. Membranes were developed using an enhanced chemiluminescence system (Amersham Life Science, Little Chalfont, UK).

**Table 1. Sequences of primers for real-time PCR.**

<table>
<thead>
<tr>
<th>Gene (accession number)</th>
<th>Forward</th>
<th>Reverse</th>
<th>Product (bp)</th>
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<tbody>
<tr>
<td>TGF-β (X52498)</td>
<td>5′-TCAAGTCAACTGTGGAAGCAA-3′</td>
<td>5′-CTCCGTCTCTTGTTGTTCA-3′</td>
<td>162</td>
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<tr>
<td>(NM_053356)</td>
<td>5′-GCATTGCGTACCTGGACGAG-3′</td>
<td>5′-GCCCTTTTTACTTATTCTGGATGCG-3′</td>
<td>197</td>
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<tr>
<td>TIMP-1 (NM_053819)</td>
<td>5′-TCCTGGTCTCCCTGGCAATA-3′</td>
<td>5′-CTGATCTGCTCCAAGCAATG-3′</td>
<td>149</td>
</tr>
<tr>
<td>(NM_053368)</td>
<td>5′-CAAGTTGAGACGTGCAGCT-3′</td>
<td>5′-AATAAACACATCGAGGCTCCA-3′</td>
<td>225</td>
</tr>
<tr>
<td>BCMO (NM_053368)</td>
<td>5′-CATCATCTCTACAGATCCCCAAAAGC-3′</td>
<td>5′-CGGGACCTGACAGACTACCTCATG-3′</td>
<td>260</td>
</tr>
<tr>
<td>β-Actin (V01217)</td>
<td>5′-CCTGGTATGC-3′</td>
<td>5′-CCCCTCTTGCTGAAGTCTC-3′</td>
<td>260</td>
</tr>
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TGF-β: transforming growth factor-β, TIMP-1: tissue inhibitor of metalloproteinase-1, BCMO: β-carotene 15,15′-monooxygenase.
CA), anti-ALDH1A2 (Santa Cruz Biotechnology Inc.), anti-ALDH1A1 (ProSci Incorporated, Poway, CA), and anti-β actin (Medical & Biological Laboratories Co. Ltd., Nagoya, Japan) were purchased for use as the primary antibodies. Anti-rat CuZn-superoxide dismutase (SOD) and Mn-SOD were provided by Dr. Keiichiro Suzuki, Hyogo College of Medicine. LRAT is a microsomal protein; hence, liver microsomes were prepared as described previously (13). For the detection of other proteins, the cytosolic fraction was prepared from the homogenate by ultracentrifugation at 100,000 g for 60 min, and the protein contents were measured using the method of Bradford (12). The extracted protein was subjected to electrophoresis, transferred to a PVDF membrane, and immunoblotted with each appropriate primary antibody. Each primary antibody was appropriately diluted with Tris-buffered saline containing Tween-20 (TBS-T), and the appropriate horseradish peroxidase-conjugated secondary antibodies were used (Bio-Rad Laboratories, Berkeley, CA). Target bands were detected using the ECL Western Blotting Detection System (GE Healthcare UK Ltd., Little Chalfont, England). The relative protein intensities were determined using ImageJ 1.46r software (National Institute of Mental Health, Bethesda, MD). The ratio of the intensity of each cytosolic protein band to that of a β-actin standard was determined, and the mean and standard deviation of the ratios were calculated.

Real-time polymerase chain reaction (PCR). Total RNA from the liver was prepared using ISOGEN (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and the protocol provided by the manufacturer. Quantitative real-time PCR was performed to determine the gene levels in the RNA samples. Reverse transcription (RT) reactions were carried out using Omniscript (Qiagen, Hilden, Germany). Subsequently, one-tenth (2 μL) of each RT reaction mixture was amplified using LightCycler PCR (F. Hoffmann-La Roche Ltd, Diagnostics Division, Basel, Switzerland) with a LightCycler FastStart DNA Master Hybridization Probe Kit or FastStart DNA Master SYBR Green I Kit (F. Hoffmann-La Roche Ltd, Diagnostics Division) according to the manufacturer's instructions. The oligonucleotide primers and accession number of genes are listed in Table 1. BCM and β-actin genes were analyzed using the Master Hybridization Probe Kit while the other genes were analyzed using the Master SYBR Green I Kit. The RT-PCR product of each gene was verified by DNA sequencing and used as the external PCR standard. Serial 10-fold dilutions of these RT-PCR products, corresponding to 1 × 10^1 copies/μL to 1 × 10^6 copies/μL were amplified in parallel with the experimental samples, as described above. By employing the LightCycler software, the amplification curves of the experimental samples were plotted against these standard curves to estimate the number of specific mRNA copies. To compensate for differences in RT efficiency among the samples, each gene value was then normalized using the β-actin copy number (14).

Statistical analysis. Results are expressed as mean ± standard deviation (SD). Comparisons were conducted using Welch’s t-tests. Differences between the groups were considered significant when p values were < 0.05.

RESULTS

Retinol and biochemical data profiles

Lipid, liver aminotransferase, and retinol levels are

<table>
<thead>
<tr>
<th></th>
<th>Control (n=6)</th>
<th>MCD (n=6)</th>
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<tbody>
<tr>
<td>Body weight (g)</td>
<td>210.8 ± 7.3</td>
<td>73.6 ± 3.7***</td>
</tr>
<tr>
<td>Total lipid (mg/dL)</td>
<td>166.3 ± 41.4</td>
<td>402.3 ± 110.2***</td>
</tr>
<tr>
<td>Triglyceride (mg/dL)</td>
<td>29.2 ± 14.1</td>
<td>70.2 ± 6.1***</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>113.0 ± 14.2</td>
<td>628.7 ± 96.2***</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>50.7 ± 19.6</td>
<td>275.4 ± 54.0***</td>
</tr>
<tr>
<td>Retinol (plasma) (μg/dL)</td>
<td>46.3 ± 4.2</td>
<td>25.5 ± 5.8***</td>
</tr>
<tr>
<td>Retinol (liver)  (μg/mg protein)</td>
<td>0.18 ± 0.04</td>
<td>0.37 ± 0.08***</td>
</tr>
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</table>

Data are expressed mean ± standard deviation (SD) and asterisks indicate significant differences (***p<0.001) between control and MCD rats.

Fig. 1. Hematoxylin and eosin (H&E) staining of the livers in control rats (A) and methionine-choline deficient (MCD) rats as a model of non-alcoholic fatty liver disease (NAFLD) (B). Magnification, ×400.
Retinol Status in MCD Rats

shown in Table 2. Hyperlipidemia and hepatic dysfunction were observed in MCD rats. The plasma retinol levels in MCD rats were significantly lower than in the controls while the hepatic retinol levels in MCD rats were markedly higher.

Hematoxylin and eosin staining, and evaluation of fibrosis

H&E staining revealed severe macrovesicular steatosis associated with foci of mild fibrosis and inflammation in the liver of MCD rats (Fig. 1). To investigate the fibrogenesis in MCD rat livers, we assessed the expression of transforming growth factor-β (TGF-β), tissue inhibitor of metalloproteinase-1 (TIMP-1), and type I procollagen mRNA using real-time PCR. Hepatic gene expression of TGF-β, TIMP-1, and type I procollagen mRNA in the MCD group was significantly higher than that in the control group (Fig. 2).

Evaluation of oxidative stress

To investigate lipid peroxidation and the antioxidant status of the liver in MCD rats, we assessed the expression of Mn-SOD and CuZn-SOD in the liver using immunoblotting and hepatic TBARS levels (Fig. 3). Expression of both Mn-SOD and CuZn-SOD were significantly lower, and the TBARS levels in the liver of MCD rats were higher than in the control group.

Expression of the BCMO gene

Figure 4 shows the results of using real-time PCR to analyze the expression of the BCMO gene in the different tissue samples. The BCMO expression was significantly

Fig. 2. Expression of rat TGF-β, TIMP-1, and type I procollagen genes obtained by real-time PCR in liver samples (n= 5/group) and analyzed as described in “Materials and Methods.” Values are reported as mean ± SD. Significant differences are indicated in the graph.

Fig. 3. Expression of rat Mn-SOD and CuZn-SOD obtained by immunoblotting (n= 3/group) and TBARS levels (n= 5/group) in rat liver samples and analyzed as described in “Materials and Methods.” Values are reported as mean ± SD. Significant differences are indicated in the graph.
lower in the intestine and liver of MCD rats than in the controls, whereas the BCMO expression in the testes and the kidneys of MCD rats was markedly higher.

Expression of retinol-related protein genes

The gene expression for retinol-metabolizing enzymes and binding proteins is shown in Fig. 5 and Fig. 6. Hepatic expression of CRBP-I, ALDH1A1, and ALDH1A2 in MCD rats was significantly higher than that in control rats (Fig. 5). Hepatic expression of LRAT in MCD rats was also significantly higher than that in control rats using both immunoblotting and real-time PCR (Fig. 6).

**DISCUSSION**

Several previous studies have evaluated retinol status in patients with NAFLD. Among Brazilian children, steatosis risk was related to retinol inadequacy (15). Moreover, a relationship has been reported between the severity of liver disease and plasma retinol levels. Circulating retinol levels in patients with liver cirrhosis or chronic hepatitis are significantly decreased (16, 17). However, Bahcecioglu et al. reported that serum retinol levels in patients with NASH and simple steatosis did not differ from those of the controls (18). These results regarding retinol status in patients with NAFLD are therefore conflicting, and further research is required. Serum retinol levels are inversely correlated with the body mass index (BMI) in morbid obesity and also inversely associated with serum transaminase levels in morbid obesity patients with NAFLD (19). NAFLD severity is inversely...
associated with retinol intake (20). These data suggest that retinol plays a critical role in liver homeostasis, and inadequate retinol status may be related to the development of NAFLD. Among adults with NAFLD, there was a significant association between insulin resistance and retinol inadequacy. In the current study, the low retinol levels in MCD rats were similar to results from human studies. This may reflect an inability to maintain liver homeostasis in the presence of NAFLD. Moreover, oxidative stress may have affected the circulating retinol levels in MCD rats, resulting in reduced hepatic expression of Mn-SOD and CuZn-SOD, and accelerated lipid peroxidation.

BCMO plays an important role in retinol metabolism. Essentially, BCMO is expressed in the liver and intestines, which are critical organs for the absorption and metabolism of β-carotene and retinol. In addition, BCMO is expressed in adipose tissue and the tissues of other organs in which retinol and retinoic acid are required for reproduction and development, such as the kidneys, lungs, testes, brain, and ovaries (21). BCMO expression is regulated by several dietary components including lipids, protein, antioxidant agents, and carotenoids in addition to retinol status (21–25). Previous studies have demonstrated that retinol status affects BCMO expression and the absorption of β-carotene. The intestine-specific homeodomain transcription factor ISX is regulated by retinoic acid through RARs (26). Moreover, the BCMO promoter in humans contains an ISX binding site, and ISX transcriptionally represses BCMO expression in the intestine (27). These findings reveal that retinoic acid negatively regulates BCMO expression via the signaling of RARs and ISX, indicating that regulation occurs in response to diet with a negative feedback mechanism (27, 28). BCMO is not only the key enzyme in retinal and retinoic acid production from β-carotene, but it is also active as a regulatory factor of lipid metabolism (29). The promoter region of human and mice BCMO genes contains a peroxisome proliferator response element, and BCMO expression is transcriptionally regulated by PPARγ, which is a member of the nuclear superfamily and has a critical role in controlling energy, lipid, and glucose metabolism (30, 31). BCMO-disrupted mice experience dysregulation of lipid metabolism and the development of hepatic steatosis (32). In the current study, BCMO expression in the liver and the intestine of MCD rats was lower than that in control rats. Repressed expression of BCMO in the liver and the intestine may lead to reduced plasma retinol levels. Furthermore, a mutually influential relationship might exist between BCMO expression in the liver and the intestine and hepatic steatosis.

In contrast, BCMO expression in the kidneys and the testes was higher than in the controls, which may have been caused by the reduced plasma retinol levels. Due to lower circulating retinol levels, the retinol metabolism in the testes and kidneys might have been controlled at the level of β-carotene conversion to retinal. Retinoids play important roles in the development and function of both the testes and the kidneys. Analysis of RAR- and/or RXR-mutant mice and experiments conducted in retinol-deficient rodents have indicated that retinoic acid is required by the testes for spermatogenesis (33). RARa/γ-mutant mice demonstrated renal agenesis and aplasia and RARa/β2-mutants demonstrated renal hypoplasia (34). Mild vitamin A deficiency in rats results in a reduced number of nephrons (35). The regulation of BCMO expression may differ among tissues that require retinoic acid and/or metabolize retinol. The differences in the BCMO gene expression patterns between tissues were reported in type 2 diabetes and obesity (24, 25). Further studies will be needed to clarify the variation in the regulation of BCMO gene expression in different tissues.

Ashla et al. compared the expression patterns of retinoid metabolism-related proteins, including bind-
ing proteins, enzymes, cytochrome P450, and nuclear receptors, in addition to the antioxidant proteins in the livers of patients with NAFLD with those in control subjects (36). They reported that the hypermetabolic state of retinoids was observed in the livers of NAFLD patients. They suggest that the catabolism of retinoic acid increases and that retinoid storage in the liver is insufficient. Similarly, the expression of CRBP1, ALDH1, ALDH2, and LRAT in the livers of MCD rats was higher than those in control rats in the current study. Retinol catabolism may be accelerated in MCD rat livers. Ashla et al. speculated that progressive degradation of retinoids reduces the storage of retinol of the liver and leads to the development of NAFLD (36). Transgenic mice expressing the dominant negative form of RARα lack retinoid signaling and therefore develop NAFLD. However, in the current study, hepatic retinol levels in MCD rats were higher than the levels in control rats. This finding may be explained by the markedly enhanced hepatic LRAT expression in MCD rats, which may have led to retinol storage in the liver despite accelerated retinol catabolism. This may then explain the differences in the liver’s retinol state that have been reported between human and experimental studies.

There are some limitations in the current study. First, MCD rats lacked the metabolic profile found in typical cases of NAFLD in humans. Second, MCD rats present decreased lipid levels of plasma, which seems to be a nutritional deficiency (37). However, plasma RBP levels in MCD rats were not changed compared with those of control rats (data not shown). This result suggests that the pathological condition of MCD rats including the altered expression of retinol-related proteins may lead to the reduced plasma retinol levels. Third, we assessed the retinol status and expression of retinol-related proteins in MCD rats at a single point after 6 wk breeding in the current study. Further studies using animal models with metabolic syndrome profiles will be required to clarify the role of retinol status in the pathogenesis of NAFLD. Moreover, it will be necessary to examine the time course of the development of hepatic histological findings, and the altered expression of retinol-related proteins in MCD rats.

In conclusion, retinol status and the expression pattern of retinol-related proteins, including binding proteins and enzymes, were altered in the MCD rat livers. Retinol catabolism may have been accelerated. Moreover, increased LRAT expression may affect the hepatic retinol levels in MCD rats. The variation in the BCMO gene expression patterns in different tissues and retinol status may have reciprocal effects. Further investigation is required to understand the regulation of retinol-related proteins expression in NAFLD. Understanding the mechanisms underlying retinol status in this condition will contribute to the knowledge of NAFLD pathogenesis.

Acknowledgments

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